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Assessment of Genetic Diversity of Pawpaw (*Asimina triloba*) Cultivars with Intersimple Sequence Repeat Markers

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Abstract. The pawpaw [*Asimina triloba* (L.) Dunal.] is a tree fruit native to many areas of the southeastern and midwestern United States. Kentucky State University (KSU) is designated as a satellite repository for *Asimina* for the U.S. Department of Agriculture (USDA), National Plant Germplasm System (NPGS). An assessment of the level of genetic diversity in cultivated pawpaw would assist in development of the future germplasm repository collection strategies for cultivar improvement. The objectives of this study were to identify intersimple sequence repeat (ISSR) markers that segregate in a simple Mendelian fashion and to use these markers to assess genetic diversity in 19 pawpaw cultivars. Leaf samples from the 34 progeny of controlled crosses (1-7-1 × 2-54 and reciprocal) and the parents were collected, DNA was extracted, and subjected to the ISSR methodology using the University of British Columbia microsatellite primer set #9. Seven primers yielded 11 Mendelian markers with either a 3:1 or 1:1 ratio that was confirmed by chi-square analysis. Analysis of genetic diversity using 10 of the ISSR markers from 19 pawpaw cultivars revealed a moderate to high level of genetic diversity, with a percent polymorphic loci P = 80 and an expected heterozygosity He = 0.358. These diversity values are higher than those reported for cultivated pawpaw using isozyme or randomly amplified polymorphic DNA (RAPD) markers, indicating that the ISSR marker methodolgy has a higher level of discrimination in evaluating genetic diversity in pawpaw and/or pawpaw has greater levels of genetic diversity than previously found.

The genus *Asimina* is the only temperate representative of the tropical Annonaceae, or Custard Apple family, and includes eight species, *Asimina triloba* (L.) Dunal., *A. parviflora* (Michx.) Dunal., *A. incana* (Bartr.) Exell., *A. obovata* (Willd.) Nash, *A. reticulata* Shuttlw. ex Chapman, *A. tetramera* Small, *A. pygmaea* (Bartr.) Dunal, and *A. longifolia* Kral, that are indigenous to North America (Kral, 1960; Peterson, 1991). The best-known species is *Asimina triloba*, the North American pawpaw, which has the largest edible fruit native to the United States (Darrow, 1975). This species is diploid [n = 2x = 18, (Bowden, 1948; Kral, 1960)], outcrosses (Willson and Schemske, 1980), and is pollinated by flies and beetles (Faegri and van der Piji, 1971). The pawpaw fruit is very nutritious (Peterson et al., 1982) and has an almost tropical aroma and smooth, custard-like texture, with flavors reminiscent of a combination of mango, banana, and pineapple (Layne, 1996; Shiota, 1991). The high fruit quality and attractive ornamental appearance give the pawpaw great potential as a commercial tree fruit or as a component in landscapes (Layne, 1996; Pomper et al., 1999).

From about 1900 to 1960, at least 56 pawpaw cultivars were selected and named (Peterson, 1991). However, fewer than 20 of these selections remain, with many being lost from cultivation through neglect, abandonment of collections, and loss of records necessary for identification (Peterson, 1991). Since 1960, additional pawpaw cultivars have been selected from the wild or developed as a result of breeding efforts of hobbyists. More than 40 cultivars are currently commercially available (Jones et al., 1998). The loss of cultivars over the last century may have led to erosion in the genetic base of current pawpaw cultivars (Huang et al., 1997).

In 1994, Kentucky State University (KSU) was designated as a satellite repository for *Asimina* preservation in the U.S. Department of Agriculture (USDA), National Plant Germplasm System (NPGS). Germplasm evaluation, preservation, and dissemination have been a high priority at KSU since that time. The repository orchards currently contain over 1700 accessions collected from the wild in 17 states and more than 40 cultivars. One of the goals of the repository is to assess levels of genetic diversity in native populations and commercially available cultivars. Another goal is to acquire unique germplasm to include in our repository collection. Such material could be useful in future pawpaw breeding efforts.

A range of molecular markers has been used in attempts to evaluate genetic diversity in pawpaw. Rogstad et al. (1991) used a minisatellite probe, M13, to determine the genetic variation in pawpaw collected in five states. Using data from one to 22 samples per population, these authors examined genetic variation at sixteen sites both within and among populations. They determined that genetic variation is very low within populations, but moderate genetic variation occurred between populations, attributed to the geographical range of the species. They concluded that the low level of genetic variation within populations might be due to clonality or inbreeding. However, inbreeding is considered to be rare in pawpaw's reproductive biology, because it is most likely self-incompatible and therefore may require out-crossing (Norman et al., 1992; Peterson, 1991). Huang et al. (1997) used

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Table 1. Genetic background of 19 pawpaw (*Asimina triloba*) selections.

Cultivar	Genetic background
1) Cales Creek	Wild seedling from Summers Co., W. Va.
2) Davis	Wild seedling from Eaton Rapids, Mich.
3) Greenriver Belle	Wild seedling from Hart County, Ky.
4) IXL	Seedling of 'Overleese' female x 'Davis' male selected in Eaton Rapids, Mich.
5) Middletown	Wild seedling from Middletown, Ohio.
6) Mitchell	Wild seedling from Iuka, Ill.
7) NC-1	'Davis' female x 'Overleese' male selected in Ontario, Canada.
8) Overleese	Seedling from Rushville, Ind.
9) PA-Golden(#1)	Seedling from G.A. Zimmerman collection selected in Amherst, N.Y.
10) Prolific	Seedling from Eaton Rapids, Mich.
11) Rebecca's Gold	Seedling from Eaton Rapids, Mich.
12) SAA-Zimmerman	Seedling from G.A. Zimmerman collection selected in Amherst, N.Y.
13) Sue	Wild seedling from Ind.
14) Sunflower	Wild seedling from Chanute, Kans.
15) Sweet Alice	Wild seedling from W. Va.
16) Taylor	Wild seedling from Eaton Rapids, Mich.
17) Taytwo	Wild seedling from Eaton Rapids, Mich.
18) Wells	Cultivated seedlings (open pollinated) from Salem, Ind.
19) Wilson	Wild seedling from Cumberland, Ky.

isozymes to evaluate the genetic diversity represented in 32 pawpaw cultivars and advanced selections from the breeding program of R. Neal Peterson of the PawPaw Foundation. These authors determined that the isozyme marker variation in cultivated pawpaw is comparable to those of other long-lived temperate woody perennials of widespread geographic range with insect-pollinated outcrossing breeding systems, secondary asexual reproduction and animal-dispersed seed, thus having a higher level of genetic diversity than Rogstad et al. (1991) had reported. Huang et al. (1997) acknowledged that the results may have been impacted by non-random selection because several of the trees studied may have been purposely selected by pawpaw enthusiasts for desirable characteristics such as large fruit size or good growth vigor. Using isozymes, Huang et al. (1998) also assessed the level of genetic diversity within wild collected pawpaw accessions at KSU and examined genetic diversity between pawpaw populations from different geographical locations. Isozymes were used to score 23 loci using 25 to 50 trees from each of nine populations. The level of genetic variation found in KSU repository accessions was similar to that found in cultivated pawpaws (Huang et al., 1997). Using 12 randomly amplified polymorphic DNA (RAPD) primers, Huang et al. (2000) identified 21 Mendelian markers and determined that the level of genetic diversity in six populations in the KSU repository collection was higher than determined for pawpaw by the same authors using isozymes (Huang et al., 1997, 1998). Huang et al. (2003) have also used additional RAPD markers for fingerprinting pawpaw cultivars and reported similar levels of genetic diversity in cultivated pawpaw, in terms of Nei's genetic diversity constant (H_e), to that reported for wild pawpaw populations by Huang et al. (2000).

Microsatellites, or simple sequence repeats (SSRs), are abundant throughout eukaryotic genomes (Kijas et al., 1995). The inter-simple sequence repeat PCR (ISSR-PCR) methodology uses a primer composed of a specific microsatellite sequence anchored at either the 3' or 5' end to amplify scorable DNA products (Gupta et al., 1994; Zietkiewicz et al., 1994). ISSR-PCR uses longer primers and has higher annealing temperatures than RAPD markers. It also has a higher degree of polymorphisms and reproducibility than do RAPD markers (Fang and Roose, 1997; Meyer et al, 1993). SSRs have become the marker system of choice for genetic diversity studies of many crops, such as apple [*Malus* ×*sylvestris* (L.) Mill. Var. *domestica* (Borkh) Mansf.] (Gianfranceschi et al., 1998) and pear (*Pyrus* L.) (Yamamoto et al., 2001), due to their abundance in the genome and high degree of polymorphism (Weber and May, 1989). ISSR-PCR, which uses the occurrence of SSRs in the plant genome, has been used successfully to characterize genetic diversity within and among plant populations (Qian et al., 2001). The primary objectives of this study were to identify ISSR markers that segregate in a simple Mendelian fashion and use these markers to evaluate the genetic diversity in commercially available pawpaw cultivars. We were also interested in comparing diversity estimates with those previously reported for pawpaw using isozymes and RAPD markers.

Materials and Methods

Plant material. Dormant cuttings were collected in mid-March from pawpaw trees located at the KSU Repository for *Asimina* species in Frankfort, Ky., placed in beakers of distilled water, and placed under fluorescent room lighting at room temperature (21 °C) to force bud break and leaf growth. Additional leaves were also collected from plants in the field in mid-April. Leaf material was collected from the 34 progeny of controlled crosses (22 from 1-7-1 x 2-54 and 12 from the progeny of the reciprocal cross) and from the individual parents 1-7-1 and 2-54. For the genetic diversity study, leaf samples were collected from 19 commercially available pawpaw cultivars spanning the native range of pawpaw (Table 1). Leaf samples of the pawpaw cultivars Greenriver Belle, Sue, and IXL were obtained from a private nursery (Nolin River Nut Tree Nursery, Nolin River, Ky.). Samples of all other cultivars were obtained from the KSU repository orchards in Frankfort, Ky.

DNA extraction. DNA was extracted from the leaves using the Promega Wizard Genomic DNA purification kit (Promega Co., Madison, Wis.). About 50 mg of leaf tissue was ground in a mortar with dry ice, and transferred to a 1.5 mL microfuge tube. Nuclei lysis solution was added (600 μL/tube), vortexed for 1 to 3 s and samples incubated for 15 min at 65 °C. Two microliters RNase A (10 mg·mL^{-1}; Sigma Chemical Co., St. Louis, Mo.) was added to each sample, and the tube incubated for 15 min at 37 °C. Samples were cooled for 1 to 5 min on ice, and 200 μL protein precipitation solution was added. Samples were vortexed for 20 s, centrifuged (15,800 g_n) for 5 min, then 750 μL of supernatant was transferred to a tube containing 750 μL 99% isopropanol. Samples were mixed by inversion and centrifuged for 5 min. Supernatants were discarded and the DNA pellets were first washed with 500 μL of 70% ethanol, next with 500 μL of 95% ethanol and finally air dried for 15 min. The DNA was solubilized in 150 μL of 1× TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer by an initial incubation at 65 °C for 1 h, then overnight at 4 °C. The samples were centrifuged for 3 min (15,800 g_n) to remove insoluble material and the supernatants were transferred to new tubes. The DNA concentration and a 260/280 nm absorbance ratio were determined using a GeneQuant RNA/DNA calculator spectrophotometer (Pharmacia Biotech, Cambridge, U.K.). All samples were stored at –80 °C until needed.

ISSR-PCR amplification. The PCR reaction mixture contained

Table 2. Single locus segregation and chi-square tests for 11 ISSR markers identified in the pawpaw (*Asimina triloba*) cross 1-7-1 x 2-54 and the reciprocal cross.

	Parents		Progeny			
	ISSR banding	ISSR	Phenotype (genotype)		Expected	
Locus	phenotype	genotype	1 (+/+ or +/–)	0 (–/–)	ratio	χ^2
UBC812-0640	1 × 0	(+/+ or +/– × –/–)	15[z] (8)[x]	19 (4)	1:1	0.53
UBC825-0760	1 × 1	(+/+ or +/– × +/+ or +/–)	22 (8)	12 (4)	3:1	2.17
UBC825-0696	1 × 0	(+/+ or +/– × –/–)	13 (4)	21 (8)	1:1	2.13
UBC841-1075	1 × 0	(+/+ or +/– × –/–)	19 (7)	15 (5)	1:1	0.53
UBC841-0480	1 × 0	(+/+ or +/– × –/–)	17 (5)	17 (7)	1:1	0.00
UBC853-1930	1 × 0	(+/+ or +/– × –/–)	18 (7)	16 (5)	1:1	0.13
UBC860-0875	1 × 0	(+/+ or +/– × –/–)	13 (4)	21 (8)	1:1	2.13
UBC860-0940	1 × 0	(+/+ or +/– × –/–)	17 (8)	17 (4)	1:1	0.00
UBC873-0480	1 × 0	(+/+ or +/– × –/–)	18 (7)	16 (5)	1:1	0.13
UBC873-0800	1 × 1	(+/+ or +/– × +/+ or +/–)	21 (8)	13 (4)	3:1	3.60
UBC880-1150	1 × 1	(+/+ or +/– × +/+ or +/–)	23 (8)	11 (4)	3:1	1.11

[z]The number of total individuals from the crosses 1-7-1 x 2-54 and 2-54 x 1-7-1 with the phenotype is shown.
[x]The number of individuals from the 2-54 x 1-7-1 cross is listed in brackets for the phenotype shown.

10 mM Tris-HCl, pH 9, 50 mM KCl, 0.1% Triton X-100, 200 μM dNTPs, 2 mM $MgCl_2$, 200 nM primer, 1.5 units of *Taq* polymerase, and 2 ng DNA template in 20 μL total volume. DNA was amplified using a GeneAmp PCR system 9700 (Perkin Elmer Applied Biosystems, Calif.). The program consisted of an initial period of 94 °C for 5 min, followed by 45 cycles of 45 s at 94 °C, 1 min at 50 °C, and 2 min at 72 °C with a final extension period of 10 min at 72 °C, followed by storage at 4 °C until use. The amplified products were electrophoresed at 4.5 $V \cdot cm^{-1}$ through a 1.4% agarose gel using a 1-kb ladder as the molecular weight standard (Promega Co., Madison, Wis.). The gels were then stained using ethidum bromide and visualized under UV light. Photographs were taken using a Kodak digital camera and digital photos were analyzed using Kodak Digital Science ID software (Eastman Kodak Company, Rochester, N.Y.).

Marker scoring and data analysis. ISSR primers (University of British Columbia, Canada, microsatellite set #9) were screened against 34 progeny of a controlled cross and parents to identify segregating polymorphisms. Segregating ISSR markers were identified by the manufacturer primer code corresponding to the primer responsible for their amplification, followed by a four digit number indicating product size in base pairs (Table 2). ISSR phenotypes were scored as being either present (1) or absent (0) and the corresponding genotypes were assigned as +/+ or +/– and –/–, respectively (Table 2). Genotypes that had a polymorphic band in at least two of three gels were scored as +/+ or +/–. Chi-square tests were used to determine the goodness-of-fit of these segregating polymorphisms to their expected Mendelian ratios (n = 34). Those with a χ^2 value of ≥3.84 were rejected.

Cultivars were screened for the presence of ISSR Mendelian markers that were identified in the segregating population (Table 2). Three replicate gels for cultivars were evaluated and scored for markers being either present (1) or absent (0). Cultivars having a specific Mendelian marker in at least two of three replicate gels were scored as having the marker present. For cultivars that had Mendelian markers in only one of three gels, an additional three gels were scored and genotypes with the marker in at least two of six gels were scored as having the marker present. Allele frequencies were indirectly estimated using the homozygous null genotypes (–/–) and corrected for dominance according to Lynch and Milligan (1994). Genetic statistics were estimated, corrected for small sample size (Nei, 1978), and used to determine the percentage of polymorphic loci (*P*) (95% criterion) and expected heterozygosity (H_e) (Miller, 1997). The level of genetic similarity among cultivars was determined by Nei's genetic distance (Nei, 1978). A dendrogram was constructed based on the matrix of the distances using unweighted pair-group mean analysis (UPGMA). Scores were entered in a matrix and analyzed with NTSYSpc software, version 2.02i (Exeter Software, Setauket, N.Y.). A similarity matrix was generated using the Dice coefficient, S = 2Nxy/(Nx + Ny), where Nx and Ny are the numbers of bands observed in clones X and Y, respectively, and Nxy is the number of bands common to both clones (Dice, 1945). The Dice values were then used to perform UPGMA cluster analysis and generate a dendrogram.

Results and Discussion

Eighty ISSR primers were screened that yielded a total of 234 amplified products. Of these 234 products, 30 polymorphic markers were found, and 11 of these segregated in a simple Mendelian fashion, as confirmed by chi-square analysis (Table 2). The 11 ISSR loci confirmed for Mendelian inheritance were used to assess genetic diversity in 19 pawpaw cultivars (Table 3). Marker UBC873-0800 had unreliable amplification and was not included in genetic diversity or cultivar separation analysis. Marker UBC860-0940 was present in all cultivars and UBC825-0760 was present in all but one cultivar (Sunflower), while the 8 remaining markers showed a high degree of polymorphism (Table 3). Isozyme marker variation analysis in cultivated pawpaw suggested genetic diversity components that are comparable to those of other long-lived temperate woody perennials of widespread geographic range with insect-pollinated outcrossing breeding systems, secondary asexual reproduction and animal-dispersed seed (Huang et al. 1997, 1998) (Table 4). Using the 10 ISSR markers, estimates of genetic diversity (P = 80% and H_e = 0.358) were higher than those based on isozymes (P = 44% and H_e = 0.172) for cultivated pawpaw, for RAPD markers for wild pawpaw accessions (P = 64% and H_e = 0.249) and cultivated pawpaw (H_e = 0.285) by Huang et al. (1997, 2000, 2003) (Table 4). However, care must be taken in comparing the ISSR genetic diversity estimates to those of Huang et al. (1997, 2000). Six additional pawpaw cultivars were included in this study that were not included in the isozyme study of Huang et al. (1997);

Table 3. ISSR markers scored in 19 pawpaw (*Asimina triloba*) cultivars.

	Cultivar[z]																		
Marker[y]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
UBC812-0640	1[x]	1	0	0	1	0	1	1	0	0	1	0	0	0	0	0	1	1	0
UBC825-0696	1	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0
UBC825-0760	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
UBC841-o480	0	1	0	1	0	0	0	1	0	1	0	1	0	0	1	1	1	0	1
UBC841-1075	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
UBC853-1930	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
UBC860-0875	0	0	1	0	0	0	1	1	0	1	1	0	0	1	0	1	0	0	0
UBC860-0940	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
UBC873-0480	0	1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	1	0	0
UBC880-1150	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1

[z]Cultivar numbers correspond to those in Table 1.
[y]Segregating ISSR markers were identified by the manufacturer primer code corresponding to the primer responsible for their amplification, followed by a four digit number indicating product size in base pairs
[x]0 = the marker was absent and 1 = marker was present in the cultivar.

however, their isozyme study included 15 advanced selections from the PawPaw Foundation breeding effort and 2 cultivars that were not included in this ISSR study. Huang et al. (2000) used 21 RAPD markers that segregated in a simple Mendelain fashion and used 6 populations (10 to 18 trees each) from 6 states in the KSU repository collection to evaluate genetic diversity. Direct comparisons of the effectiveness of all three marker systems would require the use of these marker systems with the same genotypes. However, it would appear that the 10 ISSR loci had a higher level of polymorphism than the nine isozyme markers of Huang et al. (1997). Previous studies did not support the idea that high levels of genetic variation could be maintained in *A. triloba*, due to its clonal reproductive habit and low seed set in nature (Rogstad et al., 1991). This study provides additional evidence that moderate to high levels of genetic diversity exist in *A. triloba* (Huang et al. 1997, 1998, 2000).

The ISSR markers could be useful tools in cultivar identification. Using Nei's (1978) genetic distance, 11 of the 19 cultivars were uniquely identified (Fig. 1). The ISSR based UPGMA dendrogram showing genetic similarity among 19 pawpaw cultivars using separation by Dice (1945) was very similar to the separation using Nei's (1978) genetic distance (data not shown). The ISSR separation confirms the findings of Huang et al. (1997, 2003), in that the cultivar NC-1, which has been thought to be a seedling of a 'Davis' x 'Oversleese' cross, does not share many markers with these cultivars. A mix up of pollen or seed must have occurred and NC-1 may have a different parentage than that reported. The cultivar IXL, which is thought to be a seedling of an 'Overleese' x 'Davis' cross, did show some genetic similarity with these parents. Genetic relationships for the remaining cultivars in this study were similar to those found by Huang et al. with isozymes (1997) and RAPD markers (2003). The pawpaw cultivars SAA-Zimmerman and Mitchell, Prolific and Taytwo, and Sweet Alice and SAA-Zimmerman, shared similar grouping in both the dendrograms of Huang et al. (2003) (Fig. 1). Additional ISSR Mendelian markers could further improve genetic diversity estimates in pawpaw cultivars. We

Table 4. Comparison of genetic variation of pawpaw (*Asimina triloba*) with plant species having the same characteristics.

Species characteristics	Polymorphic locus (%) (P)[y]	Expected heterzygosity (H_e)
Life form: long-lived woody perennial[z]	64.7 ± 2.7	0.177 ± 0.010
Regional distribution: widespread[z]	58.9 ± 3.1	0.202 ± 0.015
Geographic range: temperate[z]	48.5 ± 1.5	0.146 ± 0.000
Breeding system: outcrossing-animal[z]	50.1 ± 2.0	0.167 ± 0.010
Seed dispersal: animal ingested[z]	45.7 ± 3.9	0.176 ± 0.019
Mode of reproduction: sexual and asexual[z]	43.8 ± 3.7	0.138 ± 0.016
Average of all characteristics[z]	51.2 ± 8.2	0.168 ± 0.023
California cherimoya[x]	73.3	0.330 ± 0.064
California and Spanish cherimoya[w]	44.8	0.183 ± 0.044
Cultivated pawpaw (Isozymes)[v]	44.4	0.166 ± 0.048
Pawpaw wild accessions (Isozymes)[u]	43.5	0.172 ± 0.013
Pawpaw wild accessions (RAPDs)[t]	64	0.249 ± 0.022
Cultivated pawpaw (RAPDs)[s]	---	0.285 ± 0.160
Cultivated pawpaw (ISSRs)	80	0.358 ± 0.205

[z]Data from Hamrick and Godt, 1989 derived from isozyme studies. ± standard deviation.
[y]P is the percent polymorphic loci and H_e the mean gene heterozygosity, respectively.
[x]Calculated by Huang et al. (1997) from the data published by Elstrand and Lee (1987) and included other three monomorphic loci.
[w]Calculated by Huang et al. (1997) from data published by Pascual et al. (1993) and included 16 other monomorphic loci.
[v]From Huang et al. (1997).
[u]From Huang et al. (1998).
[t]From Huang et al. (2000).
[s]From Huang et al. (2003).

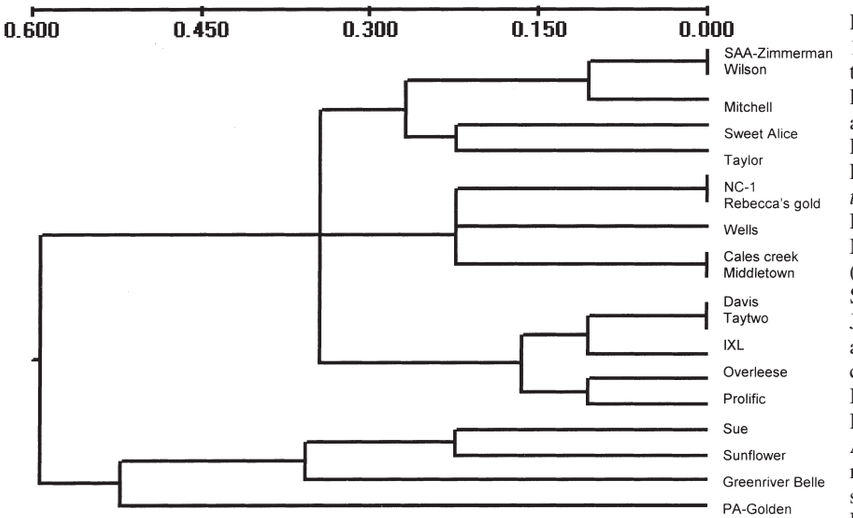

Fig. 1. ISSR based UPGMA dendrogram of Nei's (1978) genetic distance showing genetic similarity among 19 pawpaw (*Asimina triloba*) cultivars.

plan to identify additional and more reproducible ISSR markers to accurately fingerprint all pawpaw cultivars and separate remaining cultivar genetic identities.

In conclusion, seven primers yielded 11 Mendelian markers with either a 3:1 or 1:1 ratio that was confirmed by chi-square analysis. Using 10 ISSR Mendelian markers, an analysis of genetic diversity with leaf DNA samples collected from 19 pawpaw cultivars revealed a moderate to high level of genetic diversity in the selections. These data suggest that ISSR marker methodology has a higher level of discrimination in evaluating genetic diversity in pawpaw than do isozyme or RAPD marker systems.

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